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RESEARCH PAPER

Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids

Kapuganti J. Gupta[1,*], Jay K. Shah[2], Yariv Brotman[3], Kathrin Jahnke[1], Lothar Willmitzer[3], Werner M. Kaiser[4], Hermann Bauwe[1] and Abir U. Igamberdiev[2]

[1] Department of Plant Physiology, University of Rostock, Albert Einstein Str. 3, D-18059, Rostock, Germany
[2] Department of Biology, Memorial University of Newfoundland, St. John's, NL, A1B 3X9, Canada
[3] Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany
[4] Department of Botany 1, Julius von Sachs Institute of Biosciences, University of Würzburg, 97082, Würzburg, Germany

* To whom correspondence should be addressed. E-mail: jagadis.kapuganti@uni-rostock.de

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Abstract

Nitric oxide (NO) is a free radical molecule involved in signalling and in hypoxic metabolism. This work used the nitrate reductase double mutant of *Arabidopsis thaliana* (*nia*) and studied metabolic profiles, aconitase activity, and alternative oxidase (AOX) capacity and expression under normoxia and hypoxia (1% oxygen) in wild-type and *nia* plants. The roots of *nia* plants accumulated very little NO as compared to wild-type plants which exhibited ~20-fold increase in NO emission under low oxygen conditions. These data suggest that nitrate reductase is involved in NO production either directly or by supplying nitrite to other sites of NO production (e.g. mitochondria). Various studies revealed that NO can induce AOX in mitochondria, but the mechanism has not been established yet. This study demonstrates that the NO produced in roots of wild-type plants inhibits aconitase which in turn leads to a marked increase in citrate levels. The accumulating citrate enhances AOX capacity, expression, and protein abundance. In contrast to wild-type plants, the *nia* double mutant failed to show AOX induction. The overall induction of AOX in wild-type roots correlated with accumulation of glycine, serine, leucine, lysine, and other amino acids. The findings show that NO inhibits aconitase under hypoxia which results in accumulation of citrate, the latter in turn inducing AOX and causing a shift of metabolism towards amino acid biosynthesis.

Key words: Aconitase, alternative oxidase, citrate, nitrate reductase, nitric oxide.

Introduction

Nitric oxide (NO) is a versatile free radical molecule in plants, animals, and microbes (Delledonne, 2005; Besson-Bard *et al.*, 2008; Gupta *et al.*, 2011) that mediates various physiological and developmental processes. These include seed germination (Beligni and Lamattina, 2000), senescence (Guo and Crawford, 2005), stomatal closure (Desikan *et al.*, 2002), and floral transition (He *et al.*, 2004). NO is also involved in response to various stresses such as low oxygen (Dordas *et. al.*, 2003), cold (Zhao *et al.*, 2009; Gupta *et al.*, 2011), high salt (Corpas *et al.*, 2009), heavy metals (Besson-Bard *et al.*, 2009), UV-B stress (Zhang *et al.*, 2011), disease resistance (Delledonne *et al.*, 1998; Gupta, 2011), and apoptosis (Beligni *et al.*, 2002).

NO is an important regulator of plant respiration. Mechanistically, NO inhibits the respiratory chain by competitively binding to the oxygen binding sites of cytochrome *c* oxidase (COX, complex IV) (Brown, 2001). By doing so, it affects the mitochondrial electron transport chain and oxidative phosphorylation (Millar and Day, 1996; Yamasaki *et al.*, 2001). The inhibitory effect of NO on the electron transport chain is one of the reasons of its toxicity due to the suppression of respiratory oxygen uptake and generation of

superoxide resulting from over-reduction of the ubiquinone pool (Shiva *et al.*, 2001).

Besides the cytochrome pathway of mitochondrial electron transport, all plants and fungi and some protists contain an alternative respiratory pathway. This pathway comprises a single protein alternative oxidase (AOX), which in most species is encoded by a small family of nuclear genes (Millar *et al.*, 2011). The electron transfer via this protein bypasses two sites of proton translocation to the matrix and, therefore, does not lead to ATP production so the excess energy is lost as heat. AOX plays important roles when the cytochrome pathway gets inhibited under various stress conditions. The mechanism of AOX induction includes different mechanisms and could result from shifts in the level of reduced pyridine nucleotides, reactive oxygen species (ROS), or citrate (Vanlerberghe and McIntosh, 1996; Gray *et al.*, 2004), The increases in citrate and ROS act as signals to induce transcription of AOX, while pyruvate and other oxoacids are known to activate AOX allosterically at the protein level, and NADPH activates AOX via reduction of its disulphide bond (Mackenzie and McIntosh, 1999). Under conditions of hypoxia, NO is the major metabolite which can induce AOX in a similar way as shown when it was applied exogenously (Huang *et al.*, 2002), but this possibility has not been explored. While NO inhibits cytochrome oxidase (Cooper *et al.*, 1997), AOX is insensitive to NO and can provide electron flux in mitochondria even at elevated NO concentrations (Millar and Day, 1996; Gray *et al.*, 2004).

In plants, NO can be generated by various pathways and these are divided into oxidative and reductive categories. In oxidative pathways, NO is formed via oxidation of more reduced nitrogen compounds such as arginine or hydroxylamine, while in reductive pathways, NO appears as a product of reduction of nitrate and nitrite. NO synthase-like activity (Corpas *et al.*, 2009), the polyamine-mediated pathway (Tun *et al.*, 2006), and the hydroxylamine-mediated pathway (Rumer *et al.*, 2009) are oxidative pathways having limited capacity in plants and producing NO mainly for signalling purposes under normoxia (Igamberdiev and Hill, 2004; Gupta and Igamberdiev, 2011), In contrast, the nitrate reductase (NR) pathway (Sakihama *et al.*, 2002; Planchet *et al.*, 2005), the mitochondrial electron transport pathway (Planchet *et al.*, 2005; Gupta and Igamberdiev, 2011), and the plasma membrane nitrite–NO pathway (Stöhr *et al.*, 2001) are reductive pathways becoming major NO-generating mechanisms with decrease of oxygen.

The NO produced via the above-mentioned pathways under various stress conditions can induce AOX (Huang *et al.*, 2002; Fu *et al.*, 2010), which is insensitive to NO (Millar and Day, 1996). A major target of NO that has been recently identified is the enzyme aconitase (Navarre *et al.*, 2000). Aconitase contains a FeS cluster that participates in the mechanism of interconversion of three tricarboxylic acids (citrate, *cis*-aconitate, and isocitrate) and exhibits sensitivity to both NO and ROS. In plant cells, aconitase is found in both mitochondria, where it participates in the TCA cycle, and cytosol (Zemlyanukhin *et al.*, 1984), where its activity is higher and where in conjunction with NADP-dependent isocitrate dehydrogenase it participates in supplying 2-oxoglutarate for amino acid biosynthesis (Igamberdiev and Gardeström, 2003). Since the stress-related accumulation of reactive oxygen and reactive nitrogen species is particularly high in mitochondria, the inhibition of aconitase refers primarily to this compartment.

Under hypoxic conditions, NR produces high amounts of NO since the step of nitrite reduction to ammonia is inhibited and accumulating nitrite can serve as an alternative substrate for NR (Planchet *et al.*, 2005). Nitrite is also transported into mitochondria where complex IV (and possibly complex III) reduces it to NO (Gupta *et al.*, 2005). The function of hypoxically generated NO is attributed to the decrease of reduction level of pyridine nucleotides (Igamberdiev *et al.*, 2006) and limited ATP generation via the haemoglobin-NO cycle (Stoimenova *et al.*, 2007), and also to the hypoxic survival via prevention of complete depletion of oxygen (Borisjuk *et al.*, 2007; Benamar *et al.*, 2008).

The goal of the present study was to investigate the induction of AOX at low oxygen and to provide a rationale for how NO may be involved in this process. Using the wild type and the NR double mutant of *Arabidopsis*, it is demonstrated that NR is involved in hypoxically induced NO production. This NO generation correlates with the inhibition of aconitase in mitochondria which suggested that it is the increase in citrate levels that acts as a potent inducer of AOX expression. Citrate serves as AOX inductor and also as a precursor of 2-oxoglutarate for amino acid biosynthesis. The overall process involving NO production, inhibition of aconitase, and induction of AOX leads to a shift of plant metabolism towards amino acid biosynthesis.

Materials and methods

Plant material

Three-week old *Arabidopsis* seedlings from a wild type (WT) and a NR double mutant (*nia*) (obtained from National *Arabidopsis* Stock Centre, UK) in the Col-0 background were grown on vertical agar plates containing half-strength Hoagland nutrient solution containing 5 mM potassium nitrate and 2% sucrose and treated with normal air containing 21% O_2 (normoxia) or 1% O_2 (hypoxia). For determination of aconitase and NR, seedlings were transferred from the plates to liquid medium that was in equilibrium with air containing either 21% or 1% O_2.

Nitric oxide measurements

NO was measured by the gas-phase chemiluminescence detection method (Planchet *et al.*, 2005) A small glass beaker containing root slices (0.5 g root, 8 ml buffer) was immediately placed in a glass cuvette (1 l air volume) which was mounted on a rotary shaker (150 rpm). A constant flow of measuring gas (purified air or nitrogen) of 1.3 l min^{-1} was pulled through the cuvette, through a cold moisture trap, and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt, time resolution 20 s) by a vacuum pump connected to an ozone destroyer. The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (length 1 m, internal diameter 3 cm, particle

size 2 mm). Calibration was routinely carried out with NO-free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griessheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. The air temperature in the cuvette was 25 °C. NO analyser data were logged into a computer and processed using customized 'Visual designer'-based software (Intelligent Instrumentation).

Measurement of NR activity

NR activity was measured using L-NaRA-100 assay kit (NECi Co, USA). The extraction medium was 100 mM potassium phosphate buffer (1 mM EDTA, 0.4% polyvinylpyrrolidone, and 0.05% cysteine; pH 7.4). Root material (100 mg) was ground in an ice-cold mortar and pestle with 400 µl of chilled extraction buffer and centrifuged at 10,000 *g* for 10 min. The NR assay medium contained 25 mM potassium phosphate (0.025 mM EDTA; pH 7.5), 10 mM nitrate and 0.1 mM NADH. An aliquot (100 µl) of extract was added and reaction was stopped after 20 min with Zn acetate. Nitrite was determined colorimetrically as described earlier (Hageman and Reed, 1980).

Real-time quantitative reverse-transcription PCR for determination of transcript abundance of AOX1A

Total RNA was extracted from the seedlings, which were grown as indicated in the figure legends, using the RNeasy kit (Qiagen), according to the manufacturer's instructions and subjected to DNase treatment using the TURBO DNA-free kit (Ambion). RNA (5 µg) was reverse transcribed into cDNA using the Superscript III reverse transcriptase kit (Invitrogen). Real-time PCR amplification was carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems), using a power SYBR-green master mix (Applied Biosystems) and the primers described. *Ubiquitin10* (*At4G05320*) and *PDF2* (*At1g12320*) were used as housekeeping genes and the change in *AOX1A* was normalized to *Ubiquitin10*. The relative quantification of each individual gene expression of *AOX1A* was performed using the comparative threshold cycle method, as described in the ABI PRISM 7900 Sequence Detection System User Bulletin Number 2 (Applied Biosystems).

Measurement of respiration

Respiration of roots was measured using oxygen electrode (Hansatech, UK) calibrated by a simple two-point calibration (100 and 0% air saturation). Root tissue (0.1–0.2 g) were cut from the plant, washed twice and blotted on tissue paper to remove extra water, immediately cut into 4–6 mm slices, and placed in a 2.5 ml oxygen electrode chamber continuously circulated with thermostat-controlled water. To estimate the effect of citrate on the partitioning between COX and AOX and on AOX capacity, 0.2 mM citrate was fed in the medium of root cultures 3 h prior to the respiratory measurements.

Aconitase assay

Wild-type and *nia* mutant *Arabidopsis* were grown in flasks on shaker at 80 rpm for 7 days. For hypoxic treatment, the flasks were pumped with 1% oxygen for 3 h. Biomass (0.1 g) was crushed in liquid nitrogen and the enzyme was extracted in 50 mM TRIS-Cl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM citrate. Aconitase was assayed spectrophotometrically at 240 nm in the same buffer but containing 40 mM citrate (Racker, 1950). Protein concentration was estimated by the method of Bradford (1976).

GC-MS analysis

GC-MS analysis was performed as described previously (Lisec *et al.*, 2006) for six replicates each consisting of 30 pooled plants obtained from two independent experiments. Metabolite levels were determined in a targeted fashion using the Target Search software package (Cuadros-Inostroza *et al.*, 2009). Metabolites were selected by comparing their retention indexes (±2 s) and spectra (similarity >85%) against the compounds stored in the Golm Metabolome Database (Kopka *et al.*, 2005). Each metabolite is represented by the observed ion intensity of a selected unique ion, which allows for a relative quantification between groups. Metabolite data were $\log_{10}$-transformed to improve normality (Steinfath *et al.*, 2008) and normalized to show identical medium peak sizes per sample group.

Results

Nitrate reductase-dependent NO increase under hypoxia

The NR double mutant (*nia*) has a deletion in *NIA2* gene and an insertion in *NIA1* gene. This mutant was previously shown to have no detectable NR activity (Wang *et al.*, 2004). The present study could detect very low NR activity in this plant, which can be explained by a background nitrite formation via possible alternative nitrate reduction pathways (Wienkoop *et al.*, 1999). In order to check NO production rates, NO emission from the WT *Arabidopsis* root slices was measured by the gas-phase chemiluminescence method. The slices did not produce NO under normoxia (21% O_2 in 100% air saturation), while upon imposing hypoxia (1% O_2 in air saturation) NO production from roots increased and reached a steady-state rate of 6.5 nmol (g FW)$^{-1}$ h^{-1} after 30 min (Fig. 1A). To determine whether NO production in WT roots under low oxygen is due to NR, the NO production from roots of the NR double mutant (*nia*) was measured. Roots of the *nia* double mutant produced very little NO and its emission did not increase even under hypoxia (Fig. 1B). Under normoxic conditions, the total NR activity in *nia* roots was 0.026 µmol (g FW)$^{-1}$ h^{-1} whereas WT roots exhibited an NR activity of 0.099 µmol (g FW)$^{-1}$ h^{-1} which is nearly 4-times higher than in *nia* plants. In hypoxic conditions, NR activity in the WT increased to 0.22 µmol (g FW)$^{-1}$ h^{-1} in comparison to the WT under normoxic conditions (Fig. 1C), i.e. more than 2-fold, while no detectable increase was observed in the mutant. This clearly suggests that NR is involved in the hypoxic NO production either directly or by supplying nitrite for nitrite:NO reductase activity of mitochondria and possibly other compartments.

Nitric oxide inhibits aconitase and increases citrate levels

The mitochondrial form of aconitase is a constituent of the TCA cycle. Its inactivation by NO or superoxide hence decreases cellular energy metabolism and may have a protective effect against additional oxidative stress by acting as a reversible 'circuit breaker' that prevents wasteful turnover of the TCA cycle and further increases in reduction level in stress conditions (Gardner and Fridovich, 1991; Gardner *et al.*, 1997). The present study tested the effects of hypoxia and presence of NR on aconitase in plants by measuring

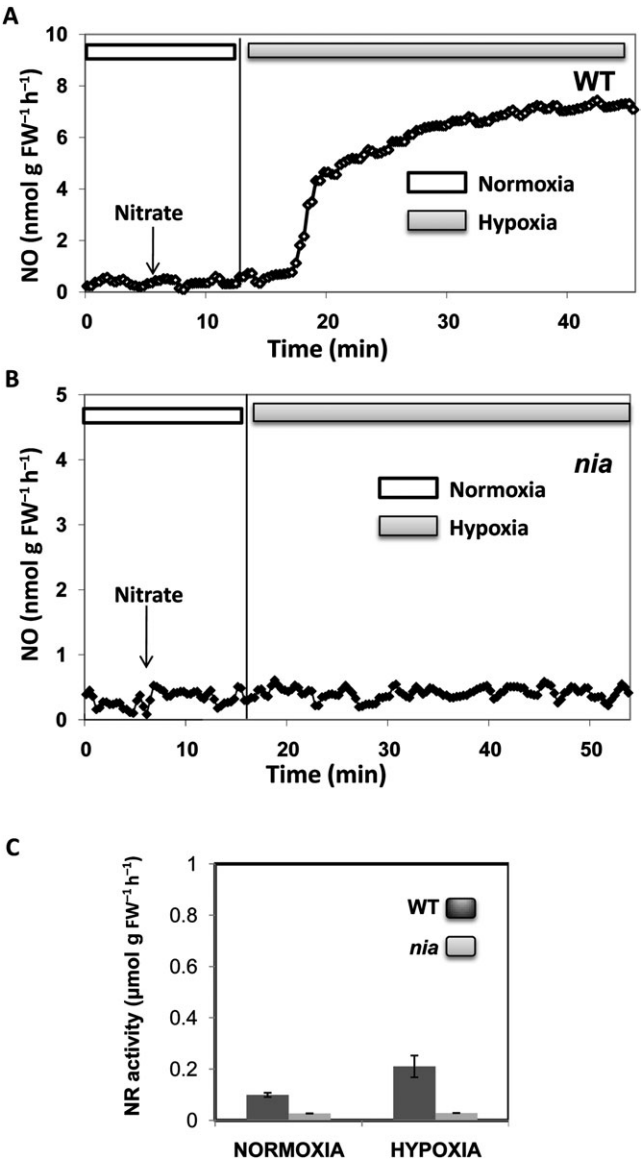

Fig. 1. Nitric oxide emissions and nitrate reductase activity in wild-type (WT) and *nia Arabidopsis* plants. (A) NO emission from WT roots under normoxia (21% O_2) and hypoxia (1% O_2): 0.5 mM nitrate was added as indicated. (B) NO emission from *nia* roots under normoxia and hypoxia. (C) Nitrate reductase (NR) activity in WT and *nia* roots under normoxic and hypoxic conditions.

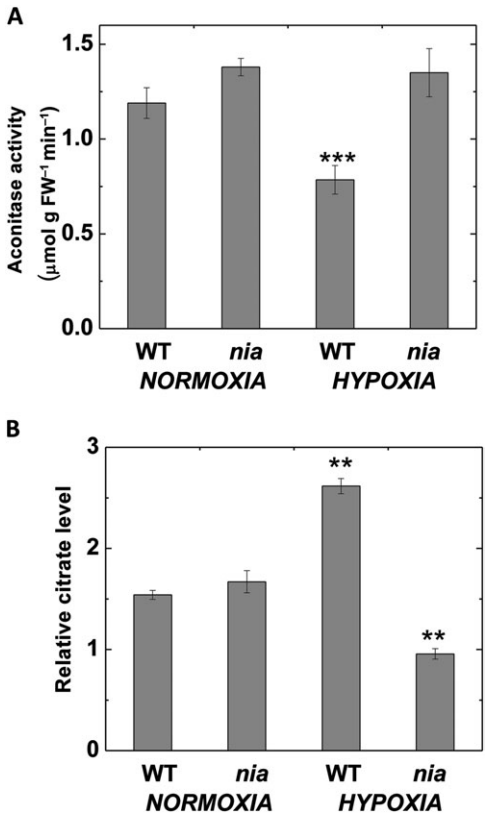

Fig. 2. Aconitase activity and citrate levels in wild-type (WT) and *nia Arabidopsis* plants under normoxia and hypoxia. (A) Aconitase activity. (B) Citrate levels measured as GC-MS values from WT and *nia* under normoxia and hypoxia. Levels are relative to WT under normoxic conditions. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.01$; *** $P < 0.001$.

aconitase activity in the WT and *nia* under normoxia and hypoxia and found that under hypoxic conditions aconitase in WT plants is strongly suppressed when compared with normoxic conditions (Fig. 2A). This is clearly in agreement with the earlier observation (Navarre *et al.*, 2000) that NO (which production is linked to NR activity, Fig. 1) is an inhibitor of aconitase. The inhibition of aconitase can lead to accumulation of citrate because aconitase mediates its conversion to isocitrate. In order to check this possibility, the citrate levels were measured by GC-MS. As shown in Fig. 2B, citrate levels were only slightly higher in the WT in comparison to *nia* under normoxic conditions. Under hypoxic conditions, while in the WT citrate level increased by 70%, in *nia* plants it was strongly (almost 2-fold) reduced. This suggests that NO inhibits aconitase and thus stimulates citrate accumulation.

AOX induction correlates with the increased levels of citrate

The involvement of the increased citrate levels in AOX induction was tested by measuring AOX and COX capacities via incubation of root slices with and without salicylhydroxamic acid, which is an inhibitor of AOX, and cyanide, which is an inhibitor for COX. The results were also verified with *N*-propylgallate (the inhibitor of AOX) and myxothiazol (the inhibitor of complex III). As shown in Fig. 3A, the total respiration (without inhibitors) in *nia* roots was 109 µmol (O_2) (g FW)$^{-1}$ h^{-1}, whereas the respiratory rate in WT roots was about 66 µmol (g FW)$^{-1}$ h^{-1}. Also, in Supplementary Fig. S1A (available at *JXB* online), the total respiratory rates of the WT and *nia* were comparable. To examine the capacity of the cytochrome pathway, the respiratory rates of WT and *nia* roots were measured in the presence of 2 mM salicylhydroxamic acid. The capacity of the cytochrome pathway was 96.7 µmol (g FW)$^{-1}$ h^{-1} in *nia*

roots whereas it was 51.7 μmol (g FW)$^{-1}$ h^{-1} in WT roots (Fig. 3B). A similar trend was observed when cytochrome capacity was measured in the presence of 100 μM *N*-propylgallate (Supplementary Fig. S1B) suggesting that the absence of NO in *nia* roots triggers operation of the cytochrome pathway with 2-times higher intensity as compared to the presence of NO in WT roots.

In order to check AOX capacity, the respiration of WT and *nia* plants was measured in the presence of 1 mM KCN (Fig. 3C). The respiratory rate in *nia* roots was 9.5 μmol (g FW)$^{-1}$ h^{-1}, whereas in WT roots the respiratory rate was 14.3 μmol (g FW)$^{-1}$ h^{-1}. A similar trend was observed when using 25 μM myxothiazol (Supplementary Fig. S1C). These results clearly suggest that in the WT, which exhibits elevated NO levels under hypoxia (Fig. 1A), AOX capacity is increased. In the absence of NO, as seen in *nia*, respiration followed the cytochrome pathway. In order to verify that the *in vivo* increase in citrate levels is responsible for AOX induction, citrate was fed to the medium of root cultures 3 h prior to the respiratory measurements. In the condition when citrate was fed to *nia* plants (Fig. 3D). AOX capacity was 18.2 μmol (g FW)$^{-1}$ h^{-1}, whereas in the absence of citrate it was 9.6 μmol (g FW)$^{-1}$ h^{-1}. These data suggest that citrate is responsible for the induction of AOX, which is in agreement with the results of Degu *et al.* (2011) showing that the inhibition of aconitase by citramalate (2-hydroxy-2-methylbutanedioate) induces AOX.

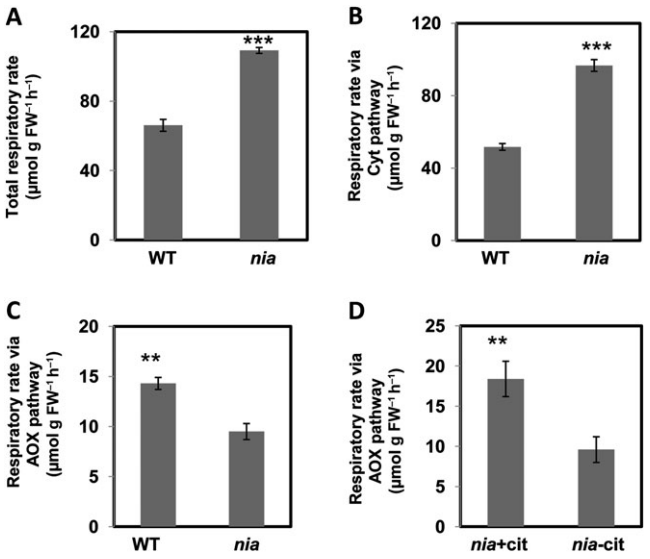

Fig. 3. Respiratory rates and capacities of the cytochrome and alternative oxidase (AOX) pathways in the roots of wild-type (WT) and *nia Arabidopsis* plants. (A) Total respiratory rates measured without inhibitors. (B) Respiratory rate in the presence of 2 mM salicylhydroxamic acid, indicating the capacity of the cytochrome pathway. (C) Respiratory rate in the presence of 2 mM KCN, indicating AOX capacity. (D) AOX capacity of *nia* plants without citrate feeding (*nia*–cit) as compared to *nia* plants fed with 0.2 mM citrate 3 h before measurements (*nia*+cit). Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.05$; *** $P < 0.005$.

Increased AOX capacity in the presence of NO is correlated with gene expression and protein biosynthesis

A possible correlation between AOX respiratory capacity and its gene expression, was estimated by measuring the transcript abundance of the AOX gene by quantitative reverse-transcription PCR. In WT, the expression of *AOX1A* was slightly higher than in *nia* in normoxic conditions, whereas under hypoxic conditions *AOX1A* gene expression was 3-fold higher (Fig. 4A). In order to check whether the transcript abundance and AOX respiratory capacity correlate with its protein amount, an immunoblot analysis was performed using polyclonal antibodies raised against *Arabidopsis* AOX. The Western blot (Fig. 4B) clearly shows that *AOX1A* has low expression in normoxic conditions in both WT and

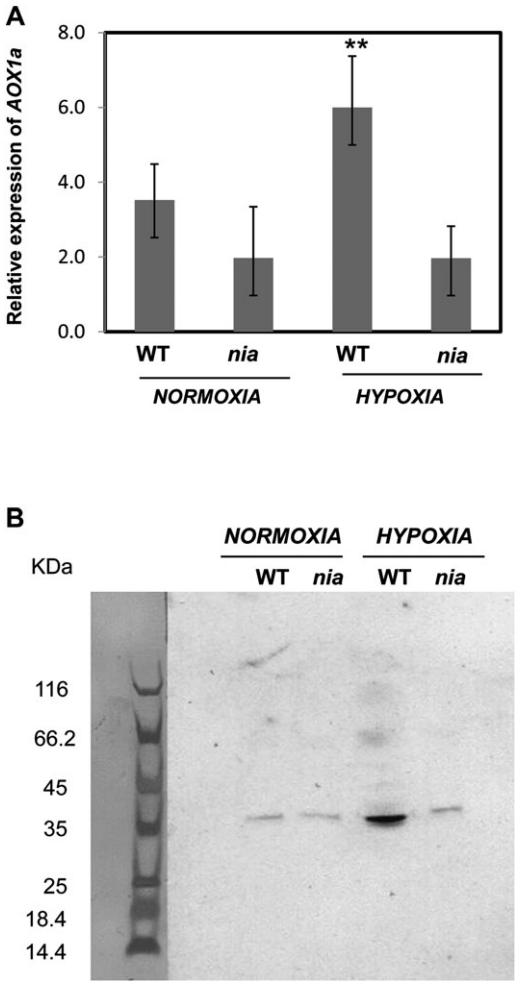

Fig. 4. Expression of alternative oxidase gene (*AOX1A*) and proteins under normoxia and hypoxia. (A) Relative gene expression of *AOX1A* in wild-type (WT) and *nia* plants under normoxic and hypoxic conditions. Error bars indicate SD. Asterisks indicate significant differences between responses to oxygen conditions: ** $P < 0.05$. (B) Immunoblotting of proteins from WT and *nia* with AOX polyclonal antibody. The 35-kDa band indicates the AOX1A protein.

nia. Under the hypoxic conditions, *AOX1A* is induced much stronger in the WT than in *nia*.

NO-induced inhibition of aconitase leads to increase in amino acid biosynthesis

The GC-MS analysis of WT and *nia* plants exposed to normoxia and hypoxia was performed for identification of the changes in various metabolites. Fig. 5 shows increased amino acid accumulation in the WT under hypoxia. The most marked increase was observed for glycine (4 times), less significant for leucine, lysine, tyrosine, arginine, ornithine, citrulline (2 times), valine, serine, methionine, histidine (~1.5 times), and only a marginal increase was observed for alanine, aspartate, and proline. Glutamate levels did not change and putrescine was slightly reduced in the WT under hypoxic conditions. The involvement of NR in the supply of amino groups for amino acid synthesis is supported by data obtained with the double NR mutant. Under normoxia, the NR deficiency did not result in a marked shift in the level of several amino acids including glycine, serine, valine, leucine, arginine, ornithine, citrulline, histidine, and tyrosine. A significant increase in glutamate and a decrease in aspartate revealed a marked shift between these two acidic amino acids. A clear decrease in alanine and proline was observed. The most striking effect of reduction in NR activity on amino acid levels was seen under hypoxia. A strikingly strong decrease was observed for all investigated amino acids, and this decrease was 5-fold or even more for several amino acids, including glycine, serine, and alanine. The hypoxic treatment also resulted in an elevation of γ-aminobutyric acid (GABA) in the WT, while in *nia* the level of GABA markedly decreased.

Changes in TCA cycle intermediates in response to NO

The data in Fig. 6 clearly show that the TCA intermediates citrate, succinate, fumarate, malate, and oxaloacetate accumulated under hypoxia in WT plants. In *nia* plants, the levels of succinate, fumarate, and malate were much lower under normoxic conditions, but the levels of citrate, 2-oxoglutarate, and oxaloacetate were kept at about the same level as in the WT. Under hypoxia in *nia* plants, a decrease was observed for all examined intermediates (except fumarate which still remained low). The 2-oxoglutarate levels in the WT were decreased under hypoxia by 20% and much increased in *nia*, but in the WT they were correlated with 60% accumulation of citrate while in *nia* the citrate levels strongly decreased under hypoxia.

Discussion

Nitrate reductase triggers amino acid metabolism under normoxia and hypoxia

The observed very low NO production in *nia* plants, which is not increased significantly even under hypoxia, clearly indicates that NR is a major source for NO production either directly by using nitrite as an alternative substrate upon its accumulation (Rockel *et al.*, 2002; Sakihama *et al.*, 2002) or indirectly by supplying nitrite for nitrite:NO reductase activity of mitochondria (Gupta and Igamberdiev, 2011) and possibly of other cell constituents such as the plasma membrane (Stöhr *et al.*, 2001). Under low oxygen, the increase of flux through NR limits the capacity of nitrite reductase (Botrel *et al.*, 1996), the activity of which can be repressed under hypoxia (Ferrari and Varner, 1971), causing a drastic accumulation of nitrite, a major substrate for reductive NO production at low oxygen tensions (Planchet *et al.*, 2005). Thus, the *nia* mutant of *Arabidopsis*, in which both genes encoding NR isoforms are affected, is indispensable in clarifying the role of NR in nitrogen and carbon metabolism, in particular in conditions of hypoxic stress, and it also reveals how the absence of NO production affects physiological processes.

The comparison of amino acid levels determined by GC-MS in wild-type and *nia* plants indicates a vital role of NR in nitrogen metabolism under oxygen deficiency. For the increase of amino acid biosynthesis, the supply of reduced nitrogen (via nitrate and nitrite reduction) should be complemented with an increased supply of carbon skeletons (via accumulation of citrate) and the latter can be achieved by the mechanism that involves inhibition of mitochondrial aconitase (Degu *et al.*, 2011) resulting in outflow of citrate from the TCA cycle.

The obtained results show that amino acid synthesis is strongly increased under hypoxia. This increase is dependent on NR activity and related to the induction of NR under hypoxia. In *Arabidopsis*, this shift does not lead to the preferential formation of alanine as it was shown for many species (Narsai *et al.*, 2011) but primarily results in a preferential formation of glycine which may be related to hypoxic accumulation of glycolate, considered as one of metabolic markers of anaerobiosis (Narsai *et al.*, 2009). While alanine is formed from pyruvate at the last step of glycolysis as an alternative to lactic and ethanolic fermentation (Rocha *et al.*, 2010), glycine formation may be linked to the deviation of glycolytic metabolism at the level of 3-phosphoglyceric acid, resulting in the non-photorespiratory serine pathway (Kleczkowski and Givan, 1988), the key enzymes of which were recently shown to be strongly upregulated under anoxia (Narsai *et al.*, 2009; Shingaki-Wells et al., 2011). This pathway may represent a major flux in stress, in particular hypoxic, conditions, leading to accumulation of glycine and glycolate and regulating another important hypoxically induced pathway of nitrogen metabolism, the GABA shunt, by supplying glyoxylate for transamination of GABA (Clark *et al.*, 2009) and by sharing the common reductase for reduction of glyoxylate and succinic semialdehyde (Allan *et al.*, 2008, 2009). The formation of GABA, according to the present data, is dependent on NR activity and increased nitrogen metabolism. The GABA shunt, which is important for pH regulation, is hypoxically induced (Fait *et al.*, 2007; Simpson *et al.*, 2010).

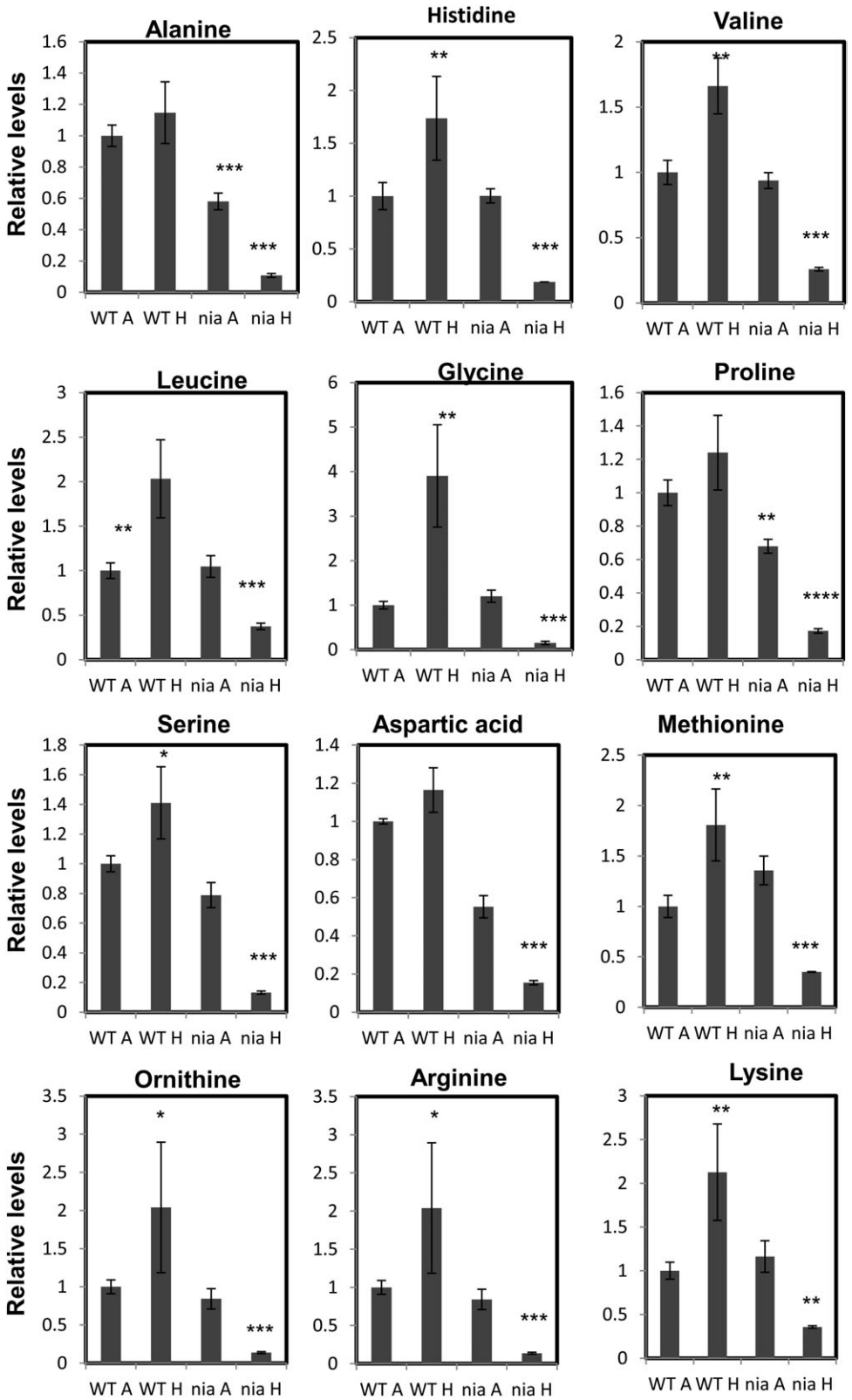

Fig. 5. Relative levels of amino acids in wild-type (WT) and *nia* plants under normoxia (A) and hypoxia (H) as measured by GC-MS. Levels are relative to WT under normoxic conditions. Asterisks indicate significant differences between responses to oxygen conditions: * $P < 0.05$; ** $P < 0.05$; *** $P < 0.005$.

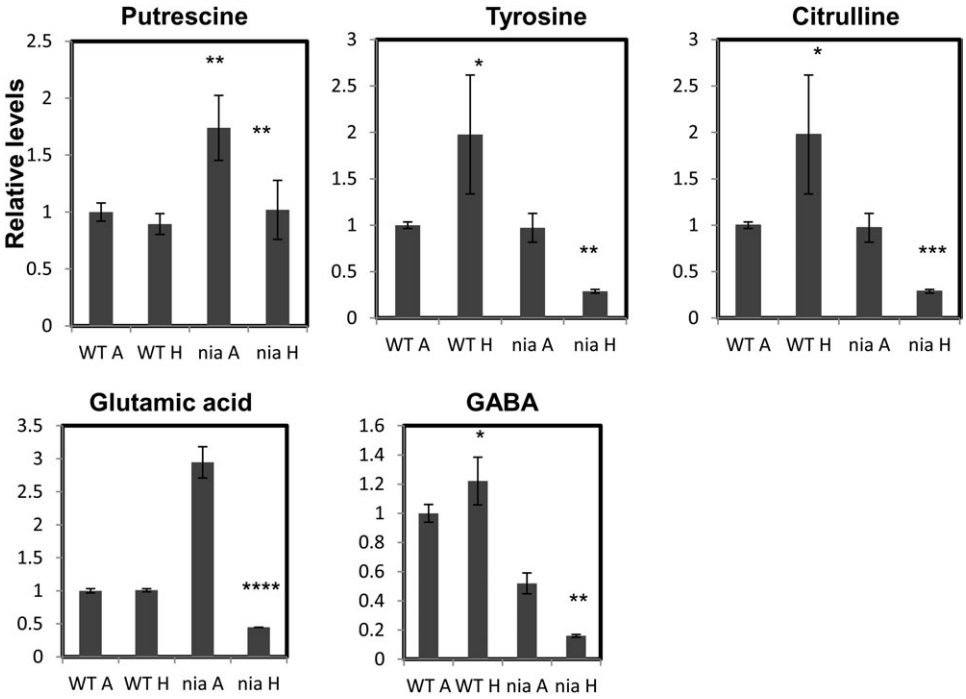

Fig. 5. Continued

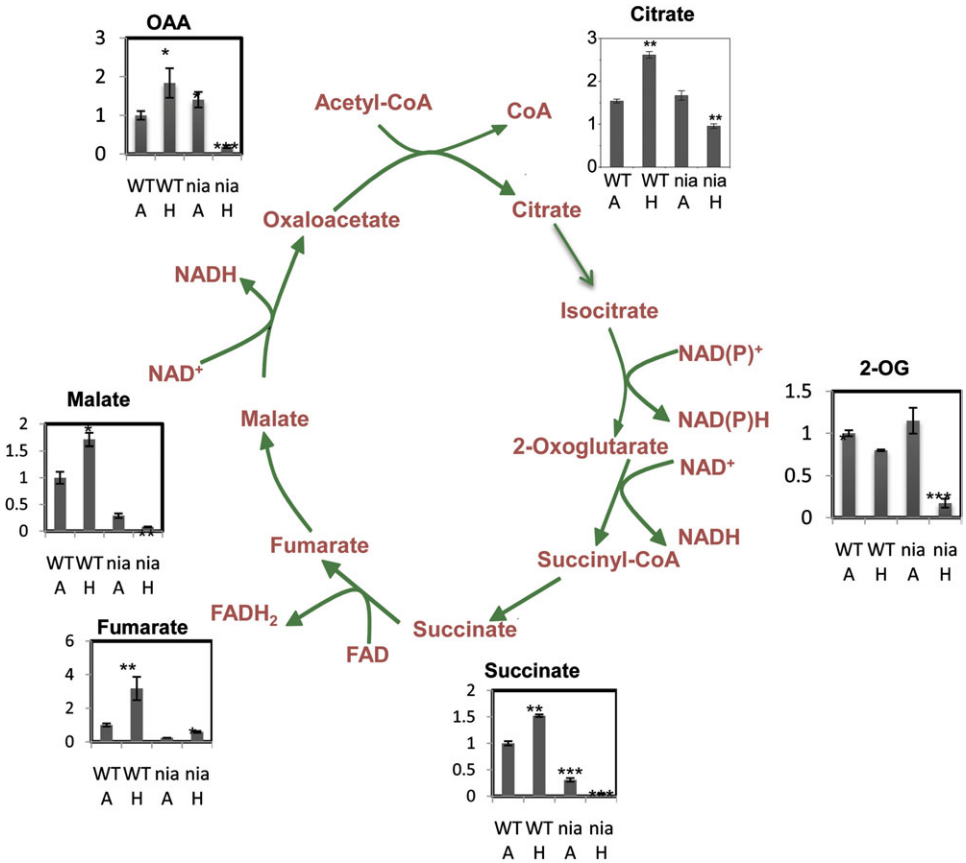

Fig. 6. Relative levels of the TCA cycle intermediates in wild-type (WT) and *nia* plants under normoxia (A) and hypoxia (H) as measured by GC-MS. OAA, oxaloacetate; 2-OG, 2-oxoglutarate. Levels are relative to WT under normoxic conditions. Asterisks indicate significant differences between responses to oxygen conditions: * $P < 0.05$; ** $P < 0.05$; *** $P < 0.005$.

The reductive pathway of NO formation under hypoxia

Nitrate and nitrite reductases can provide ammonium for both the serine biosynthetic pathway and the GABA shunt, while NO, being a co-product of nitrate/nitrite reduction, can facilitate ammonium assimilation by diverting carbon from the TCA cycle by inhibiting the mitochondrial aconitase. While ammonium formation results from complete reduction of nitrate, the incomplete reduction of nitrate (to NO) may induce a mechanism for supplying carbon skeletons to ammonium incorporation to form amino acids. The data on TCA cycle intermediates, in particular the accumulation of citrate in wild-type plants under hypoxia as compared to *nia* plants, support this suggestion. Another role of NO in such a system will consist in prevention of over-reduction and participating in some build up of ATP. NO can indirectly support metabolism via turnover of the haemoglobin-NO cycle (Igamberdiev and Hill, 2004). In this cycle, NO generated from nitrite by mitochondria is converted (oxygenated) to nitrate which in turn is supplied to NR and reduced again to nitrite. This cycle helps to prevent over-reduction of the hypoxic cell and contributes to a limited ATP synthesis (Stoimenova *et al.*, 2007), which can support assimilation of ammonium.

Under hypoxic conditions, NO originates from nitrate (Planchet *et al.*, 2005) in a reductive process that involves nitrite:NO reductase activity of mitochondria (Gupta and Igamberdiev, 2011), of other organelles such as the plasma membrane (Stöhr *et al.*, 2001), or of NR reductase itself (Rockel *et al.*, 2002; Sakihama *et al.*, 2002). The oxidative pathway of NO formation (Corpas *et al.*, 2009) will be inhibited at low oxygen. The existence of this pathway in plants, however, is still under debate (Moreau *et al.*, 2008, 2010) because neither a NO synthase gene nor a protein has been identified as yet. Therefore, most likely, the hypoxically accumulated nitrite is responsible for observed high NR activity and high rates of NO emission (Fig. 1C). The cytosolic acidification taking place at low oxygen further stimulates the NR (Kaiser and Brendle-Behnisch, 1995) and the NO production (Rockel *et al.*, 2002; Planchet *et al.*, 2005) Even if the major part of NO is formed in hypoxic roots by mitochondria as suggested (Planchet *et al.*, 2005), these organelles depend on NR for nitrite to produce NO.

Reliable methods are important for measurement of NO production in plants. Various methods have been developed for the NO measurement; however, there are numerous limitations for using the diaminofluorescent dye-based assays (Planchet and Kaiser, 2006; Mur *et al.*, 2011). The present measurements used the most reliable chemiluminescence method in which NO reacts with ozone and produces a chemiluminescence signal. Moreover, all plants were grown on sterile cultures to exclude any microbial interference in the assays.

A link between NO, aconitase, and AOX

Aconitase contains FeS clusters that are sensitive to hydrogen peroxide (Verniquet *et al.*, 1991) and NO (Navarre *et al.*, 2000) and, through this, aconitase is involved in modulation of the resistance to oxidative stress and in apoptosis (Moeder *et al.*, 2007). A strong inhibition of aconitase by NO is stronger at lower pH (Gardner *et al.*, 1997), which is commonly observed under hypoxia. The stronger inhibition of aconitase in the WT but not in NR mutant (*nia*) suggests that NO is directly responsible for the irreversible inhibition of aconitase under hypoxia.

In response to a range of stress factors, the induction of the alternative pathway resulting from high AOX activity is thought to keep ROS levels in check by increasing the capacity of mitochondrial electron transport and diverting electrons from reduced ubiquinone to oxygen, thus preventing formation of superoxide (Maxwell *et al.*, 1999). While at the protein level AOX is activated by pyruvate and by the increased reduction level (Mackenzie and McIntosh, 1999), the most powerful inducer of AOX at the gene level is citrate (Vanlerberghe and McIntosh, 1996). This may establish the link between aconitase, the enzyme of the TCA cycle that is most sensitive to reactive oxygen and nitrogen species, and the component of electron transport chain which can effectively prevent formation of reactive oxygen and cope with elevated NO levels.

The results provide substantial evidence that AOX is induced under hypoxic conditions via citrate production caused by the inhibition of aconitase by nitric oxide. AOX may lower the ROS produced during low oxygen (Blokhina *et al.*, 2001) and during re-oxygenation following hypoxia (Skutnik and Rychter, 2009). The induction of AOX can serve as a priming mechanism to counteract the anticipated increased ROS production during reoxygenation (Maxwell *et al.*, 1999). Retrograde signalling that senses the metabolic state of the organelle and transduces that signal to the nucleus to express corresponding genes can be achieved via inhibition of aconitase by NO leading to elevation of citrate, the latter acting as a signal for the induction of the AOX gene in the nucleus. The observed correlation between citrate accumulation and 2-oxoglutarate decrease in wild-type (but not *nia*) plants is in favour of the suggested regulatory chain. Therefore, induction of AOX by citrate is a part of retrograde signalling. Such induction was shown to be associated also with the active photorespiratory flux which increases the redox level in mitochondria and stimulates nitrogen metabolism. The plants of barley (Igamberdiev *et al.*, 2001) and potato (Bykova *et al.*, 2005) with low expression of the glycine decarboxylase complex are characterized by a very low expression of AOX protein. Since both photorespiration and hypoxia are linked to active nitrogen metabolism, AOX may be involved in the evolutionary adaptation of plants as a part of the mechanism supporting metabolic switch to intensive nitrogen assimilation.

The observed increase in amino acid accumulation in wild-type *Arabidopsis* under hypoxia and the lack of effect in the NR mutant showing the involvement of NR in amino acid production can be facilitated by inhibition of aconitase by NO, causing the supply of citrate for providing carbon skeletons for amino acid synthesis. Operation of mitochondria and their electron transport in these conditions becomes

possible due to the induction of AOX by accumulating citrate, which supports a limited flux through mitochondria that contributes to formation of 2-oxoglutarate for ammonium incorporation into amino acids and to a limited ATP synthesis (Gupta and Igamberdiev, 2011). Thus, under hypoxia, a retrograde interorganellar signalling pathway is induced in which NR activation triggers NO formation, which in turn inhibits aconitase and increases the level of citrate, which serves as an AOX inducer. This pathway leads to a shift of plant metabolism towards amino acid biosynthesis and provides an important adaptation strategy of plants to low oxygen conditions.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Respiratory rates and capacities of the cytochrome and AOX pathways in the roots of wild-type and *nia Arabidopsis* plants.

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